The prognostic role of MYC structural variants identified by NGS and FISH in multiple myeloma

Running title: MYC structural variants in multiple myeloma

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Statement of translational relevance

Although structural variants (SVs) involving *MYC* are common in multiple myeloma (MM), the prognostic significance of different *MYC* SVs has not been clearly established. Here we evaluated the significance of *MYC* SVs in MM detected by NGS and FISH. *MYC* SVs were common and 9 different subtypes could be identified by NGS. Only non-Ig insertion and Ig insertion subtypes showed significant differences in outcome. Non-Ig insertion was associated with improved outcome, while the Ig insertion subtype (specifically IgL) was associated with reduced outcome. Although FISH is commonly used to detect *MYC* SVs, FISH failed to identify ~70% of *MYC* SVs. *MYC* SVs detected by FISH were more likely to be associated with higher *MYC* gene expression and poor outcome. Genome wide NGS approaches should be considered as a replacement technique for a more comprehensive evaluation of the tumor clone, in comparison to traditional cytogenetic methodologies such as FISH.
Abstract

Purpose: Structural variants (SV) of the MYC gene region are common in multiple myeloma (MM) and influence disease progression. However, the prognostic significance of different MYC SVs in MM has not been clearly established.

Experimental design: We conducted a retrospective study of MM comparing MYC SV subtypes identified by next generation sequencing (NGS) and fluorescence in situ hybridization (FISH) to MYC expression and disease survival using 140 cases from Mayo Clinic and 658 cases from the MMRF CoMMpass study.

Results: MYC SVs were found in 41% of cases and were classified into 9 subtypes. A correlation between the presence of a MYC SV and increased MYC expression was identified. Among the 9 MYC subtypes, the non-Ig insertion subtype was independently associated with improved outcomes, while the Ig insertion subtype, specifically involving the IgL gene partner, was independently associated with poorer outcomes compared to other MYC SV subtypes. Although the FISH methodology failed to detect ~70% of all MYC SVs, those detected by FISH were associated with elevated MYC gene expression and poor outcomes suggesting a different pathogenic role for FISH-detected MYC subtypes compared to other MYC subtypes.

Conclusion: Understanding the impact of different MYC SVs on disease outcome is necessary for the reliable interpretation of MYC SVs in MM. NGS approaches should be considered as a replacement technique for a more comprehensive evaluation of the MM clone.
Introduction

Multiple myeloma (MM) is an incurable malignancy of plasma cells (PCs) with ~32,000 new cases in the US each year (1,2). Genetic abnormalities of MM contribute to disease heterogeneity and influence response to therapy and prognosis (1). Primary genetic abnormalities, characterized by recurrent immunoglobulin (Ig) heavy chain (IgH) structural variations (SV) and/or hyperdiploidy resulting from multiple trisomies, occur early in disease course, while secondary genetic abnormalities such as 1q gain, 17p deletion resulting in loss of TP53 and SVs involving the MYC proto-oncogene occur upon disease progression (3). Genetic events t(11;14)(q13;q32) CCND1/IgH, t(6;14)(p21;q32) CCND3/IgH and hyperdiploidy are associated with standard risk, whereas t(4;14)(p16.3;q32) FGFR3/MMSET/IgH, t(14;16)(q32;q23) IgH/MAF, t(14;20)(q32;q12) IgH/MAFB, 1q gain and TP53 deletions and single nucleotide variants (SNVs) are associated with high risk (3).

Although genetic abnormalities involving MYC have been documented in the progression from smoldering multiple myeloma (SMM) to MM (4-6), the prognostic role of MYC SVs has not been fully established in the context of MM. Earlier studies showed MYC SVs detected by fluorescence in situ hybridization (FISH) or target capture-based sequencing were independently associated with poor outcome (7-10). However, more recent studies using whole genome sequencing (WGS) did not uniformly support this finding (excluding poor outcome MYC/IgL SVs) (4,11-13). This discrepancy is likely due to differences in methods and sensitivities of detection of MYC SVs by FISH or target capture-based sequencing compared to WGS. Given that MYC SVs often display remarkable genomic heterogeneity with numerous gene partners, reduced detection of MYC SVs by FISH is not unexpected (4,8,9,11,12,13). This possibility is supported by lower frequencies of MYC SVs found by FISH (~13-15%) compared to next generation sequencing (NGS) (~23-42%) consistent with a high false-negative rate of the MYC break apart (BAP) FISH probe (15). To more fully understand the role of MYC SVs in
MM disease outcome, we performed a retrospective study to compare the MYC SV subtypes identified by FISH and NGS to the expression of MYC and overall disease survival.

Materials and Methods

This was an institutional review board (IRB) approved retrospective study that included newly diagnosed MM (NDMM) cases from both the Mayo Clinic and publicly available Multiple Myeloma Research Foundation (MMRF) CoMMpass cases.

Mayo Clinic cohort

The Mayo Clinic (Rochester, MN campus) cohort included 1342 unique patients with MM seen within 90 days from diagnosis in the period from January 2006 to January 2018. Cytogenetic analysis by FISH, including the MYC BAP, was performed within 1 year from diagnosis and less than 6 months from the start of first-line treatment. All patients were identified using a prospectively maintained database; additional clinical and laboratory data were obtained by review of electronic medical records. All patients had authorized the use of their electronic medical record data for research. Patient samples were collected with written informed consent in accordance with recognized ethical guidelines and IRB approval. Among those patients, 140 patients had simultaneous data for both FISH and NGS using mate pair sequencing (MPseq).

Multiple Myeloma Research Foundation (MMRF) CoMMpass cohort

The MMRF CoMMpass cohort (clinical trial identifier: NCT01454297) included 658 cases with either tumor long-insert WES, WGS, RNA sequencing (RNAseq) for gene expression (IA15 release) and clinical outcome data (IA16 release). The study was approved by ethics committees or IRBs at the study sites. All patients provided written informed consent in accordance with recognized ethical guidelines. RNAseq data from the CoMMpass cohort are displayed as Salmon TPM and log2 transformed [log2(MYC TPM+0.25)-log2(0.25)] values.
Fluorescence in situ hybridization (FISH)

FISH analysis was performed on bone marrow (BM) samples as previously described (16,17) and further described in supplemental methods.

DNA extraction and mate pair sequencing (MPseq)

DNA extraction and MPseq library preparation methods have been previously described (18-20) and further described in supplemental methods.

Bioinformatics and visualization

A total of 658 CoMMpass cases and 200 normal peripheral blood samples with WGS data were analyzed for breakpoint junction (SV) and copy number abnormality (CNA). The sequencing FASTQ files were aligned to GRCh38 reference genome using BWA-MEM 0.7.17 (21). The output BAM was processed to determine coverage (reads/Kb) across the genome and normalized using a GC content and mapability (Umap k24) (22) correction and then binned to 30Kb windows. Regions of similar copy number level were segmented using a sliding window method and then the copy number value of each region was normalized by the mode of the coverage probability distribution function in order to center the values around the expected 2N level. CNA regions were calculated as any region that deviated from this expected 2N level by >10% (loss <= -10% deviation, gain >= +10% deviation). CNA of TP53 and CKS1B were identified as any region that deviated from this expected 2N level by ≤1.74 as loss and >2.3 as gain to call TP53 deletions and 1q gains. For SV detection, reads that mapped to locations ≥5Kb bp apart or to different chromosomes were considered discordant. These discordant fragments were clustered by both fragment size (absolute difference in genomic positions) and midpoint (sum of genomic position). Clustering was done on both parameters using a cutoff of 5Kb. The clusters from the normal peripheral blood WGS samples were used to create a 5Kb mask to eliminate likely false positive from the clustering results. A further filtering was applied...
that required junction calls to have \( \geq 3 \) supporting fragments to be called and have a genomic footprint of \( \geq 50 \text{bp} \) on both sides of the junction. These SV and CNA calls were combined and visualized in a genome U-plot (19), which allowed us to interpret and characterize the \textit{MYC} alterations into distinct structural motifs. \textit{MYC} SV partner frequencies were calculated with R (https://www.R-project.org) and plotted using the ggplot2 library (23) or Circos (24).

**Receiver operator curve (ROC)**

The ROC curve (25) was created by plotting the false positive rates vs. true positive rate using RNA expression level (Transcription per Million: TPM) and \textit{MYC} alterations were classified using WGS data from the 658 samples from the CoMMpass cohort.

**Statistical analysis**

All statistical analyses were performed using statistical software JMP (Version 14.1.0, SAS Institute Inc., Cary, NC), SPSS15 (Chicago, IL, USA) or GraphPad Prism software (San Diego, CA) and significance were determined at \( p < 0.05 \). Categorical variables were compared using the chi-square test. A non-parametric (Kruskal-Wallis test) and post hoc (Dunn) test was used to determine difference between more than two sample groups. Overall survival (OS) was defined as time from diagnosis to death from any cause or to last follow up, with those alive censored at date of last follow up. Progression-free survival (PFS) was defined as the time from initiation of first-line treatment until progression or death from any cause, or last follow up (Mayo Cohort) or from time of diagnosis until progression or death from any cause, or last follow up (CoMMpass cohort). Survival curves were estimated using Kaplan Meier and compared using the Log-Rank test. Where survival probability did not reach 0.5, mean survival times were calculated.

**Data availability**

The MPseq data that supports this study have been deposited in the National Center for Biotechnology Information (NCBI)’s Sequence Read Archive with BioProject ID PRJNA739382

Results

Characterization of MYC SV subtypes

A total of 658 cases from the CoMMpass cohort with both WGS and MYC expression data were included, and the associated abnormality impacting the MYC gene region was investigated. Forty-two cases (6.4%) had a CNA involving MYC without a MYC SV, including 18 cases (2.7%) with monosomy 8 (one copy of the MYC gene) and 24 cases (3.6%) with trisomy 8 (3 copies of the MYC gene) (Table 1). Nineteen cases (2.9%) with a deleterious SNV impacting the MAX gene were included in a separate category given the extremely low MYC gene expression as previously reported (4) (Table 1). We found 327 cases (49.7%) had neither a MYC SV, CNA, nor deleterious MAX SNV and 270 cases (41.0%) had evidence of an SV impacting the MYC gene region. We further categorized the cases with a MYC SV into 9 subtypes based on the type of MYC SV observed (Table 1, Supplemental Figure 1). These included cases with terminal tandem duplications (TTD) (duplication of the genomic segment downstream of MYC) (n=29, 4.4%), terminal deletions (terminal del) (deletion of the genomic segment downstream of MYC) (n=11, 1.7%), proximal deletions (proximal del) (deletion of the genomic segment upstream of MYC) (n=13, 2.0%), translocations involving immunoglobulin (Ig) (IgH, IgK or IgL) enhancer sequences (Ig translocation) (n=13, 2.0%), templated insertions of non-Ig enhancers (non-Ig insertion) (n=65, 9.9%), translocations involving non-Ig enhancer sequences (non-Ig translocations) (n=16, 2.4%), complex deletions or gains (complex del/gain) (n=30, 4.6%), templated insertions of Ig (IgH, IgK or IgL) enhancer sequences (Ig insertion) (n=88, 13.4%), and amplifications of MYC (n=5, 0.8%) (Table 1). Of 270 cases with a MYC SV, 37.4% involved an Ig enhancer and 62.6% involved a non-Ig enhancer. From 658 cases, 356...
(54.1%) were hyperdiploid without evidence of a translocation to CCND1, CCND3, MAF, MAFB or FGFR3/MMSET. Similar to other studies (4,7,9,11,12,14,26), an increase in the prevalence of hyperdiploidy in cases with a MYC SV was observed (n=191, 70.7%), compared to cases without a MYC abnormality (n=165, 42.5%). Specifically, cases with TTD, non-Ig insertion and Ig insertion had an enrichment of hyperdiploidy (Table 1).

We compared the SVs surrounding only the MYC gene region of the 9 MYC subtypes to other established patterns of SVs recently described in human cancers, including MM (27,28,29) (Supplemental Table 1). The tandem duplication (TTD) and deletion (terminal and proximal dels) subtypes were described as simple events with a single SV connecting two DNA breaks. Most (n=26, 89.7%) non-Ig and Ig translocations were categorized as balanced or unbalanced translocation events with no more than 2 chromosome partners. Most (n=148, 96.7%) non-Ig and Ig insertions were categorized as templated insertions, some being simple and others having evidence of complexity (Supplemental Table 1). The complex del/gains subtype involved non-Ig partners and had the most evidence of a complex rearrangement surrounding the MYC gene. Within this subtype, 14 (46.7%) had ≥10 SVs and these were categorized as chromothripsis similar to the definition used in (29). Of the remaining 16 complex del/gain cases with 3-9 SVs around the MYC region, a single case was classified as having chromoplexy and the remaining 15 cases (50.0%) were termed complex, not otherwise specified. We also identified 4 (4.5%) Ig insertions subtypes as having chromothripsis and a single case as being complex, not otherwise specified (Supplemental Table 1). Three (23.1%) of the Ig translocation subtypes were also described as complex. Amplifications were defined as cases where the estimated copy number of MYC was ≥7N. The 5 MYC amplifications were achieved by either chromothripsis (n=4) or aneuploidic gain or double minute (n=1) (Supplemental Table 1). Of 270 cases with a MYC SV, the most common SV category was templated insertion (54.8%) (Supplemental Table 1). If eliminating simple SVs (TTD and
terminal and proximal dels), the frequency of templated insertion was 68.2% of cases, similar to that reported in (29).

Of 365 total MYC SVs identified in 270 cases with a MYC SV, there were 146 unique partner genes (Figure 1A, Supplemental Table 2, Supplemental Figure 2A-C). Although the most common gene partner involved enhancers of the following genes, IgH at 14q32.33 (13.7%), IgL at 22q11.22 (9.9%), NSMCE2 at 8q24.13 (8.5%), TXNDC5 at 6p24.3 (7.1%), IgK at 2p11.2 (5.5%), FAM46C (TENT5C) at 1p12 (3.0%) and CSMD3 at 8q23.3 (2.2%), recurrent partners were found in association with specific subtypes (Supplemental Table 3). The complex del/gain subtype was enriched for NSMCE2 and CSMD3 partners and the proximal dels involved mostly NSMCE2. Although each Ig insertion case at minimum included IgH, IgL or IgK, 55.7% of the cases (49/88) had 3 or more partners (including MYC) in the “templated insertion chain” involving non-Ig genes; the most common being TXNDC5. Of the 49 Ig insertion cases with multiple partners, 8 (16.3%) did not have a direct connection between the Ig and MYC gene regions, but rather were separated by a non-Ig insertion in between (5 IgH, 1 IgK, and 2 IgL). TXNDC5 was the most common MYC partner in the non-Ig insertion subtype. The most common gene partner in non-Ig translocations was FAM46C. Non-Ig insertion subtype was also commonly associated with 3 or more partners within the “templated insertion chain” (Supplemental Table 3-4). No clear MYC partner was identified in TTD and terminal del subtypes. Similar to Mikulasova, et al, (12), 57.1% of cases had 2 chromosome partners, 23.6% had 3, 12.1% had 4 partners and 7.1% had 5 or more partners (Supplemental Table 4). In contrast to the high frequency of MYC SVs, only 8 (1.2%) cases had a non-synonymous MYC SNVs with a median MYC expression of 42.9 TPM, with 6 co-occurring in cases that also had a MYC SV (Supplemental Table 5).

Type of MYC SV and association with MYC gene expression
We next compared MYC RNA levels in each MYC SV subtype. MYC RNA levels have previously been shown to correlate with the expected MYC gene signature (genes directly targeted by MYC) and a MYC gene signature has also been shown to correlate with MYC protein levels (30,31). These findings suggest that MYC RNA levels are functionally associated with the predicted effect of MYC transcription. MYC expression correlated with the presence of a MYC SV using ROC curve analysis plotting the false positive vs. true positive rate for the classification of samples for MYC alterations using MYC TPM data. The AUC value of 0.823 suggests strong correlation of MYC expression with the presence of a MYC SV (Figure 1B). Significant differences in MYC expression were also observed in some MYC SV subtypes compared to cases without a MYC SV (Figure 1C, Table 1, Supplemental Table 6). Lowest median MYC expression was observed in cases with a MAX SNV (1.1 TPM) or monosomy 8 (10.3 TPM). The 327 cases with no MYC SV had a median MYC expression of 21.6 TPM. Compared to cases with no MYC SV, expression of MYC was significantly increased in cases with proximal del (55.9 TPM), non-Ig insertion (68.0 TPM), non-Ig translocation (70.8 TPM), complex del/gain (75.0 TPM), Ig insertion (86.0 TPM), and amplification (144.1 TPM) and was significantly reduced in cases with a MAX SNV (Figure 1C, Table 1, Supplemental Table 6). Significant differences in MYC expression were not identified between cases with no MYC SV and monosomy 8, trisomy 8, TTD, terminal del and Ig translocations and between many of the individual MYC SV subtypes (Supplemental Table 6).

**Type of MYC SV and detection by FISH**

Given the heterogeneity of the MYC SVs identified by NGS and our previous observation of reduced detection of MYC SVs by MYC BAP FISH compared to NGS (15), we evaluated which MYC SVs give an abnormal MYC BAP FISH result using our Mayo Clinic cohort with MPseq and FISH data (n=140). Similar to Abdallah, et al (16), 7 cases (5.0%) of cases were abnormal using the MYC BAP FISH probe while 41 cases (29.3%) were abnormal by MPseq. Using MPseq and FISH data described in Smadbeck, et al. (15), subtypes more likely to be
undetected by FISH include proximal deletions and non-Ig and Ig insertions (Supplemental Table 7, Supplemental Figure 3).

We next analyzed 648 CoMMpass cases with the goal to infer, based on visualization of the genomic architecture from WGS and knowledge of the location of the MYC BAP FISH probes, whether a MYC SV could be detected by FISH. Using this approach, we predicted a MYC SV could be detected by FISH in 82/658 (12.5%) of cases, similar to the ~13-15% detected using MYC FISH in previous studies (32,33). We predicted the MYC BAP FISH probe would miss ~69.6% of all MYC SVs and would have reduced sensitivity in detecting TTD, terminal deletions, non-Ig insertions and Ig insertions (Supplemental Table 8, Figure 1D). FISH was predicted to detect most amplifications, complex deletion/duplications, most non-Ig and Ig translocations and some non-Ig and Ig insertions (Supplemental Table 8, Figure 1D).

Of cases with hyperdiploidy, 75.4% had a MYC SV predicted to be missed by FISH. Cases with MYC SVs predicted to be missed by FISH had a slightly lower MYC gene expression (63.7 TPM, range 5.2-611.5 TPM) compared to cases predicted to be detected by FISH (80.1 TPM, range 8.9-1111.8 TPM) (p=0.119) (Supplemental Table 8).

**MYC gene expression and patient survival**

We next evaluated whether elevated MYC gene expression, irrespective of MYC SV status, was associated with differences in patient survival. Boxplot analysis was used to categorize MYC expression in 631 CoMMpass cases with available OS and MYC expression data. Top quartile/high MYC expression was defined as MYC expression ≥75.0 TPM and bottom quartile/low MYC expression was defined as ≤16.5 TPM. Although mean overall survival (OS) and progression free survival (PFS) was shorter in patients with high MYC expression compared to patients with low MYC expression (OS: 4.6 years vs. 5.3 years, p=0.038, PFS: 2.5 years vs. 3.1 years, p=0.068) (Figure 2A-B), the findings were only significant for OS. Using MYC expression as a continuous variable, high MYC expression was associated with an increased risk of death on univariate analysis (p=0.027), but this was not considered significant.
in a multivariate model including MYC expression and other high risk abnormalities such as TP53 deletion, 1q gain, high risk translocations, t(4;14), t(14;16) and t(14;20), ISS stage III and ≥70 years of age (Table 2). Similar observations were observed when analyzing MYC as a categorical variable (high vs. low/medium MYC expression) in the full cohort (Supplemental Table 9). High MYC expression was significantly associated with inferior OS in cases classified as non-hyperdiploid (p=0.001), but not in cases classified as hyperdiploid (p=0.843) (Supplemental Figure 4).

Although median age was similar among cases with high MYC expression (64 years) compared to low MYC expression (63 years), cases with high MYC expression had an increased frequency of TP53 deletion (14.5%), 1q gain (48.0%) and ISS stage III (36.0%) compared to cases with low MYC expression (8.0% with TP53 deletion, 24.1% with 1q gain, 25.4% ISS stage III). Low MYC expression was associated with a low frequency of any MYC SV (8.6%), including MYC/IgL SVs (1.1%) (Figure 2C, Supplemental Table 10). Of cases with a MYC SV present in the low MYC expression group, 3.2% were TTD, 2.1% were non-Ig insertion, 0.5% had a complex del/gain and 1.1% had an Ig insertion. In contrast, 80.2% of cases in the high MYC expression group had evidence of a MYC SV. The most common MYC SV in this group included Ig insertion (33.8%), with 35% of these Ig insertion cases involving IgL. Twenty-six cases (17.2%) with high MYC expression had no MYC abnormality detected (Figure 2C, Supplemental Table 10).

**MYC SVs detected by FISH, but not by NGS, are associated with poor survival outcome**

We compared the impact of MYC SVs on patient survival when detected by FISH or NGS. We recently reported that disease survival was shorter in patients with a MYC SV compared to patients without a MYC SV using the MYC BAP FISH probe (5.3 vs. 8.0 years, p<0.001) (16). Among patients with hyperdiploidy, OS was decreased in patients with a MYC SV detected by FISH compared to those without a MYC SV (5.7 vs. 8.6 years, p=0.007) (Figure 3A). Similarly, among patients without hyperdiploidy, OS was decreased in patients with a MYC SV compared to those without a MYC SV (5.3 vs. 8.0 years, p<0.001) (16).
SV detected by FISH compared to those without a MYC SV (2.8 vs. 6.8 years, p<0.0001) (Figure 3B). However, among patients with hyperdiploidy, PFS was similar to those with a MYC SV detected by FISH compared to those without a MYC SV (4.4 vs. 4.3 years, p=0.680) (Figure 3C). In contrast, among patients without hyperdiploidy, PFS was decreased in those with a MYC SV detected by FISH compared to those without a MYC SV (2.6 vs. 3.5 years, p<0.001) (Figure 3D).

In contrast, there was no significant difference in OS and PFS between patients from the Mayo cohort with a MYC SV detected by MPseq compared to those without a MYC SV (OS: 7.7 vs. 6.9 years, p=0.990 and PFS: 2.5 and 3.0 years, p=0.490) (Figure 3E-F). Similarly, there was no significant difference in OS and PFS between patients from the CoMMpass cohort with a MYC SV detected by WGS compared to those without a MYC SV (OS: 5.7 and 5.5 years, p=0.415, PFS: 3.3 and 3.1 years, p=0.755) (Figure 3G-H). Although no significant differences in OS and PFS between patients with a MYC SV and those without a MYC SV were observed, a trend towards improved OS and PFS was identified in the hyperdiploid cohort in contrast to the non-hyperdiploid cohort (Supplemental Figure 5).

Although evaluation of all MYC SVs together resulted in no significant differences in OS and PFS compared to cases with no MYC SVs, the impact of individual MYC SVs subtypes on OS and PFS was further evaluated. Among the 9 MYC SV subtypes, only non-Ig insertion and Ig insertion subtypes showed significant differences in either OS and/or PFS in univariate analysis (Supplemental Table 11). Patients with non-Ig insertion subtype had improved OS and PFS, while patients with Ig insertion had reduced OS and PFS (Figure 4, Supplemental Figure 6). Non-Ig insertion subtype was associated with improved OS (RR 0.33, p=0.004) and PFS (RR 0.60, p=0.018), while Ig insertion subtype was associated with reduced PFS (RR 1.46, p=0.016) on univariate analysis (Supplemental Tables 12-13). Among the Ig insertion subtype, only those involving the IgL gene partner, were associated with reduced PFS (RR 2.12, p<0.001) on univariate analysis (Supplemental Table 14). In a multivariate model including...
non-Ig insertion and the favorable risk category of hyperdiploidy, non-Ig insertion retained its 
prognostic value at predicting OS and PFS (OS RR 0.32, p=0.005, PFS RR 0.60, p=0.025) 
(Supplemental Table 12). In a multivariate model including either Ig insertion or IgL SVs (all IgL 
insertions) and other high risk abnormalities, both Ig insertion and IgL SVs retained their 
prognostic significance at predicting PFS (Ig insertion, RR 1.40, p=0.042, IgL, RR 2.29, 
p<0.001) (Supplemental Tables 13-14), with a trend towards reduced OS and PFS in Ig 
insertion subtypes with increased MYC partners (Supplemental Figure 7).

Discussion

Structural variants involving the MYC proto-oncogene are common secondary events in MM and are associated with SMM to MM disease progression (4-6,8,9,11-14). We observe 
MYC SVs in 41% of MM cases detected by WGS and similar to other studies, WGS identified a 
greater frequency of MYC SVs compared to FISH (4,11-13). Among the 9 MYC SV subtypes, 
the non-Ig insertion and Ig insertion subtypes, namely IgL insertions, showed significant 
differences in outcome in comparison to cases without a MYC SV, similar to other studies 
(4,11,12). The non-Ig insertion subtype was associated with improved outcomes, while the Ig 
insertion subtype (specifically when partnered with IgL) was associated with poor outcomes. 
Thus, MYC appears to be associated with poor outcome only when partnered with IgL. The 
main driver is likely the association with IgL as IgL SVs, even in the absence of partnering with 
MYC, are associated with poor outcome (11). The poor outcome associated with IgL SVs has 
been proposed to involve the strong association of the IKZF1 transcription factor to the IgL locus 
rendering cases with IgL SVs less sensitive to IMiD treatment (11). How the non-Ig insertion 
subtype confers a prognostic benefit independent of hyperdiploidy remains unknown.

Within clinical genomics laboratories, MYC SVs are most often identified using BAP 
FISH probes spanning the MYC genomic region. FISH detects ~13-15% of MYC SVs in newly 
diagnosed MM (32,33), compared to ~23-42% detected by WGS (4,11-13). FISH
underestimates the frequency of MYC SVs in MM by about 2-3 fold (4,8,12,15), likely due to the heterogeneity of MYC SV breakpoints. This makes the design of FISH probes capable of capturing all MYC SVs a significant challenge. Similar to MM, ~32% of DLBCL were also discordant between FISH and a hybrid capture sequencing assay (34). Most of these discordant cases had non-Ig partners with breakpoints outside of the genic cluster (34). Most of the cases with non-Ig SVs represented high-grade B cell lymphomas, a WHO entity that requires correct MYC classification for accurate diagnosis and patient management. Understanding the limitations of the MYC BAP probe is critical since clinical laboratories use FISH for the evaluation of MYC SVs and evidence of false negative MYC BAP FISH results have been reported (15,35-38). We show that FISH has reduced sensitivity towards non-Ig and Ig insertions subtypes because these rearrangements often occur between the 5’ and 3’ MYC FISH probes and do not always result in a separated 5’ and 3’ FISH pattern. Although the MYC FISH probe fails to detect ~70% of MYC SVs that are detected by NGS, the MYC probe can still identify MYC SVs in ~12% of MM patients. MYC SVs detected by FISH have a higher gene expression and are more likely to be associated with poor outcome suggesting a different pathogenic role compared to other MYC SVs.

Older studies using FISH or target capture-based sequencing found that MYC SVs were associated with inferior survival (7-10). Another study identified MYC SVs in 13% of MM patients with no correlation with other primary abnormalities and no prognostic impact on survival (33). Whether these findings are explained by differences in MYC probe placement or differences in the proportion of hyperdiploidy to non-hyperdiploidy is uncertain. Other studies also found no difference in outcome between cases with or without MYC SVs detected using WGS. Although MYC SVs are more common in hyperdiploidy (4,7,9,11,12,14,26), the influence of MYC SVs on survival in this group has been unclear. While previous studies have shown that FISH-detected MYC SVs are associated with poor outcome only in the hyperdiploid group (7),
our findings suggest that FISH-detected MYC SVs and also high MYC expression may be more predictive of poor outcome in the non-hyperdiploid group. It is possible that differences in the frequency and ratio of the favorable non-Ig insertion to the unfavorable Ig insertion subtype between hyperdiploid and non-hyperdiploid groups (Supplemental Figure 8) may explain some of the differences in outcomes between these cohorts when evaluating MYC SVs.

Previous studies have shown that MYC expression levels correlate with an expected MYC gene signature (30). A MYC signature has also been shown to correlate with MYC protein levels detected by IHC (31), providing support that MYC RNA levels are associated with the predicted effect of MYC transcription. MYC overexpression using IHC has been identified in ~40% of MM patients, similar to the frequency of MYC SVs identified in this study, and was associated with reduced survival (39,40). We also observe that increased MYC expression was independently associated with poorer OS, but primarily in non-hyperdiploid cases. These differences may be explained by the reduced frequency of the non-Ig insertion subtype in the non-hyperdiploid group. We also observed 26 cases (4%) had high MYC expression without evidence of a MYC SV. This finding may be due to epigenetic factors, other SNVs, a MYC SV not detected by NGS or by increased signaling from a pathway such as NF-kB that promotes MYC expression (41). Elevated BIRC3, NFKB2 and NFKBIA expression was observed in cases with high MYC expression without a MYC SV (data not shown), consistent with a linear relationship between MYC expression and NF-kB index in cases without a MYC SV (4).

Although high MYC expression in this subtype with no MYC SV showed a trend toward reduced survival, the sample size was small and results were not considered significant (p=0.234) (data not shown). While expression microarray or RNAseq has been proposed in the evaluation of MM (42), this approach has not been uniformly applied into clinical practice and the relative instability of RNA compared to DNA based genetic tests can be problematic. Evaluation of MYC protein levels by IHC has also not always correlated with the presence of MYC SVs detected by
FISH raising the concern that IHC may not be sensitive enough to function as a surrogate for MYC SVs (43); a finding that is not surprising given the challenges of detecting MYC SVs by FISH.

This study is limited by its retrospective nature and by the heterogeneity in treatment regimens in both cohorts. In addition, our sample size for cases with MPseq results was limited, however the results were consistent with the much larger CoMMppass cohort. Another limitation is the lack of available MYC FISH data and the reliance on inferring the FISH result from our training set that included NGS and FISH data from 70 previous cases from Smadbeck, et al (15).

In summary, we show that non-Ig insertion MYC subtypes are associated with improved outcomes and Ig insertion MYC subtypes (specifically those involving IgL) are associated with poor outcomes. Although the MYC BAP FISH approach has the potential to miss nearly 70% of MYC SVs demonstrating that NGS appears to be a more robust technique to characterize a greater fraction of MYC SVs, FISH can identify MYC SVs associated with higher gene expression and poorer outcome. In addition to identifying a greater fraction of MYC SVs, NGS has the potential to identify other clinically significant SVs, CNAs, and SNVs that are currently not routinely evaluated in clinical genomics laboratories. Although NGS is currently associated with increased costs, longer assay turn-around-times, greater analysis complexities and increased data storage requirements compared to FISH (15), NGS approaches should be evaluated as a replacement technique for a more comprehensive evaluation of the tumor clone, in comparison to traditional cytogenetic methodologies such as FISH.

Acknowledgments

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References


Table 1: MYC subtype and expression.

<table>
<thead>
<tr>
<th>MYC subtype</th>
<th>Median raw TPM</th>
<th>Median Log transformed TPM</th>
<th>Total (frequency in cohort)</th>
<th>Total hyperdiploidy (frequency in subtype)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No MYC abnormality</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No MYC SV</td>
<td>21.6</td>
<td>6.4</td>
<td>327 (49.7%)</td>
<td>142 (43.4%)</td>
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<tr>
<td>MAX SNV</td>
<td>1.1</td>
<td>2.4</td>
<td>19 (2.9%)</td>
<td>2 (10.5%)</td>
</tr>
<tr>
<td>Monosomy 8</td>
<td>10.3</td>
<td>5.4</td>
<td>18 (2.7%)</td>
<td>5 (27.8%)</td>
</tr>
<tr>
<td>Trisomy 8</td>
<td>35.9</td>
<td>7.2</td>
<td>24 (3.6%)</td>
<td>16 (66.7%)</td>
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<tr>
<td>TTD</td>
<td>38.3</td>
<td>7.3</td>
<td>29 (4.4%)</td>
<td>22 (75.9%)</td>
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<tr>
<td>Terminal del</td>
<td>44.1</td>
<td>7.5</td>
<td>11 (1.7%)</td>
<td>5 (45.5%)</td>
</tr>
<tr>
<td>Proximal del</td>
<td>55.9</td>
<td>7.8</td>
<td>13 (2.0%)</td>
<td>7 (53.8%)</td>
</tr>
<tr>
<td>Ig translocation</td>
<td>61.5</td>
<td>7.9</td>
<td>13 (2.0%)</td>
<td>9 (69.2%)</td>
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<tr>
<td>Non-Ig insertion</td>
<td>68.0</td>
<td>8.1</td>
<td>65 (9.9%)</td>
<td>55 (84.6%)</td>
</tr>
<tr>
<td>Non-Ig translocation</td>
<td>70.8</td>
<td>8.1</td>
<td>16 (2.4%)</td>
<td>5 (31.3%)</td>
</tr>
<tr>
<td>Complex del/gain</td>
<td>75.0</td>
<td>8.2</td>
<td>30 (4.6%)</td>
<td>17 (56.7%)</td>
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<tr>
<td>Ig insertion</td>
<td>86.0</td>
<td>8.4</td>
<td>88 (13.4%)</td>
<td>69 (78.4%)</td>
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<tr>
<td>Amplification</td>
<td>144.1</td>
<td>9.2</td>
<td>5 (0.8%)</td>
<td>2 (40.0%)</td>
</tr>
<tr>
<td>Total</td>
<td>33.4</td>
<td>7.1</td>
<td>658 (100%)</td>
<td>356 (54.1%)</td>
</tr>
</tbody>
</table>

MYC abnormality subtype, median MYC TPM values, total frequency of the subtype in the CoMMpass cohort (n=658) and percentage of hyperdiploidy in each MYC subtype. Overall p-value (No MYC vs. MYC subtypes) determined by Kruskal-Wallis test. Individual p-values determined by a post-hoc Dunn test (Supplemental Table 6).
Table 2: The influence of MYC expression, TP53 deletion, 1q gain, high risk translocations, ISS stage, patient age and hyperdiploidy on OS and PFS.

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Univariate Model (OS)</th>
<th>Multivariate Model (OS)</th>
<th>Univariate Model (PFS)</th>
<th>Multivariate Model (PFS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR</td>
<td>P-value</td>
<td>Lower 95%</td>
<td>Upper 95%</td>
</tr>
<tr>
<td>MYC expression</td>
<td>1.002</td>
<td>0.027</td>
<td>1.000</td>
<td>1.003</td>
</tr>
<tr>
<td>Continuous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP53 deletion</td>
<td>1.736</td>
<td>0.008</td>
<td>1.152</td>
<td>2.617</td>
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<tr>
<td>Yes vs. no</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1q gain</td>
<td>1.677</td>
<td>&lt;0.001</td>
<td>1.246</td>
<td>2.259</td>
</tr>
<tr>
<td>Yes vs. no</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High risk translocations</td>
<td>1.416</td>
<td>0.066</td>
<td>0.978</td>
<td>2.051</td>
</tr>
<tr>
<td>Yes vs. no</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISS III vs I/I</td>
<td>2.134</td>
<td>&lt;0.001</td>
<td>1.571</td>
<td>2.896</td>
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<tr>
<td>Age Group ≥ 70 vs. &lt;70</td>
<td>2.259</td>
<td>&lt;0.001</td>
<td>1.676</td>
<td>3.044</td>
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</table>

Comparison of MYC expression as a continuous variable in full cohort on overall survival and progression free survival in two models (model 1: evaluation of high risk abnormalities, model 2: evaluation of hyperdiploidy). TP53 deletion yes vs. no, 1q gain (greater than 2 copies of 1q including the CKS1B gene) yes vs. no and high risk IgH translocations (t(4;14), t(14;16) or t(14;20)) yes vs. no, ISS stage III vs. I/II and ≥ 70 years vs. <70 years of age at diagnosis. P-value determined using Wald test. Risk ratio for MYC indicated as unit risk ratio, per unit change in regressor. Risk ratios that are considered significant (p<0.05) are indicated in red. RR: Risk ratio. N=545 for univariate OS, N=523 for multivariate OS, N=542 for univariate PFS, N=521 for multivariate PFS.
**Figure legends:**

**Figure 1:** *MYC* abnormality subtypes, partner genes and *MYC* gene expression. A. Bubble plots for *MYC* SV partners. Gene name (x axis) is plotted by chromosome (y axis). No SV involving chromosome Y were detected. SV frequencies are indicated with circle size, and SV group by color (see legend). B. Receiver Operating Characteristic Curve for *MYC* Alteration Classification from *MYC* Expression. Receiver operating characteristic (ROC) curve plotting the false positive rate vs. true positive rate for the classification of samples for *MYC* alterations using mRNA expression levels in Transcripts per Million (TPM). Dotted lines gives AUC=0.5 for an uninformative classifier. C. Boxplot and median *MYC* transcript levels (log transform of Salmon TPM) of MMRF CoMMpass cases (n=658), with observed structural variants (SVs). Significant differences in each subtype were compared to the subtype with no MYC SV. *** p<0.0001, ** p<0.001, * p<0.05. D. Pie charts showing the distribution of the *MYC* SV in association with predicted FISH detection.

**Figure 2:** Impact of *MYC* gene expression on overall survival (OS) and progression free survival (PFS). A. A comparison of OS (years) in patients with low *MYC* expression (≤16.5 TPM) (blue line), medium *MYC* expression (16.5-75 TPM) (green line) and high *MYC* expression (≥75.0 TPM) (red line) detected by RNAseq from the CoMMpass cohort. OS time (mean) was 5.3 years [95%CI:5.0-5.7], 4.8 years [95%CI:4.6-5.1], 4.6 years [95%CI:4.2-5.0] in low (n=158), medium (n=314) and high (n=159) *MYC* expression cohorts, respectively. B. A comparison of PFS (years) in patients with low *MYC* expression (≤16.5 TPM) (blue line), medium *MYC* expression (16.5-75 TPM) (green line) and high *MYC* expression (≥75.0 TPM) (red line) detected by RNAseq from the CoMMpass cohort. PFS time (median) was 3.1 years [95%CI:2.4-3.9], 2.9 years [95% CI:2.4–3.3] and 2.5 years [95% CI:1.7-3.0] in low (n=158), medium (n=311) and high (n=159) *MYC* expression cohorts, respectively. C. Pie charts showing the distribution of the *MYC* SV in association with *MYC* gene expression.
Figure 3: Impact of MYC SV detected by FISH or NGS on overall survival (OS) and progression free survival (PFS). A comparison of OS (years) in patients with a MYC SV detected by FISH (blue curve), and patients without MYC SV (red curve) among patients with hyperdiploidy in A or among patients without hyperdiploidy in B from the Mayo Cohort. OS time (median) in the hyperdiploid group was 5.7 [95%CI: 5.0-6.4] years (n=79) and 8.6 [95%CI: 7.5-9.3] years (n=694) MYC SV and in no MYC SV groups, respectively. OS time (median) in the non-hyperdiploid group was 2.8, [95%CI: 1.5-6.1] years (n=33) and 6.8 [95%CI: 6.2-8.8 years] (n=504) MYC SV and in no MYC SV groups, respectively. A comparison of PFS (years) in patients with a MYC SV detected by FISH (blue curve), and patients without MYC SV (red curve) among patients with hyperdiploidy in C or among patients without hyperdiploidy in D from the Mayo Cohort. PFS time (median) in the hyperdiploid group was 4.4 [95%CI: 2.9-5.5] years (n=79) and 4.3 [95%CI: 3.7-4.9] years (n=694) MYC SV and in no MYC SV groups, respectively. PFS time (median) in the non-hyperdiploid group was 2.6 [95%CI: 1.1-2.7] years (n=33) and 3.5 [95%CI: 3.1-4.5 years] (n=504) MYC SV and in no MYC SV groups, respectively. E. A comparison of OS (years) in patients with MYC SV detected by MPseq (blue curve) and patients without a MYC SV detected by MPseq (red curve) from the Mayo cohort. OS time (median) was 7.7 [95%CI: 4.8-not reached] years (n=41) and 6.9 [95%CI: 4.8-11.2] years (N=99) in the 2 groups, respectively. F. A comparison of PFS (years) in patients with MYC SV detected by MPseq (blue curve) and patients without MYC SV detected by MPseq (red curve) from the Mayo cohort. PFS time (median) was 2.5 [95%CI: 1.9-3.4] years (n=41) and 3.0 [95%CI: 2.1-3.5] years (n=99) in the 2 groups, respectively. G. A comparison of OS (years) in patients with a MYC SV detected by WGS (blue curve) and patients without a MYC SV detected by WGS (red curve) from the CoMMpass cohort. OS time (mean) was 5.7 [95%CI: 5.3-6.1] years (n=225) and 5.5 years [95%CI: 5.2-5.9] (n=320) in the MYC SV and in no MYC SV groups, respectively. H. A comparison of PFS (years) in patients with MYC SV detected by WGS (blue curve) and patients without a MYC SV detected by WGS (red curve) from the
CoMMpass cohort. PFS time (median) was 3.3 years [95%CI:2.8-3.9] (n=224) and 3.1 years [95%CI:2.3-3.7] (n=318) in the MYC SV and in no MYC SV groups, respectively.

Figure 4: Impact of different MYC SVs detected by NGS on overall survival (OS) and progression free survival (PFS). A. A comparison of OS (years) in patients with no MYC abnormality (no MYC SV, MAX SNV, monosomy 8 and trisomy 8) (green line), patients with non-Ig insertion (blue line), patients with IgL rearrangements (all IgL insertions) (red line) and all others (orange line) detected by WGS from the CoMMpass cohort. OS time (mean) was 5.5 years [95%CI:5.2-5.9] (n=320), 6.9 [95%CI:6.3-7.4] years (n=55), 4.3 [95%CI:3.2-5.3] years (n=28) and 5.4 [95%CI:4.9-5.9] years (n=142) in the no MYC abnormality, non-Ig insertion, IgL and other group, respectively. B. A comparison of PFS (years) in patients with no MYC abnormality (no MYC SV, MAX SNV, monosomy 8 and trisomy 8) (green line), patients with non-Ig insertion (blue line), patients with IgL rearrangements (all Ig insertions) (red line) and all others (orange line) detected by WGS from the CoMMpass cohort. PFS time (median) was 3.2 years [95%CI:2.4-3.9] (n=318), 4.8 [95%CI:3.6-6.1] years (n=55), 1.4 [95%CI:1.2-1.6] years (n=28) and 3.1 [95%CI:2.6-3.5] years (n=141) in the no MYC abnormality, non-Ig insertion, IgL and other group, respectively.
Figure 2

A  OS - CoMMpass cohort

B  PFS - CoMMpass cohort

C  MYC expression group

- Total
- Low ≤ 16.5 TPM
- Medium >16.5-<75.0 TPM
- High ≥75.0 TPM

Legend:
- TTD
- Terminal del
- Proximal del
- Ig translocation
- Non-Ig insertion
- Non-Ig translocation
- Complex del/gain
- Ig insertion
- Amplification
- Trisomy 8
- Monosomy 8
- MAX mutation
- No MYC SV
Figure 3

**A** OS-Mayo cohort-hyperdiploid

- FISH detected
  - No MYC abnormality
  - MYC SV
- n=694
- n=79
- p=0.007

**B** OS-Mayo cohort-non-hyperdiploid

- FISH detected
  - No MYC abnormality
  - MYC SV
- n=504
- n=33
- p<0.0001

**C** PFS-Mayo cohort-hyperdiploid

- FISH detected
  - No MYC abnormality
  - MYC SV
- n=694
- n=79
- p=0.680

**D** PFS-Mayo cohort-non-hyperdiploid

- FISH detected
  - No MYC abnormality
  - MYC SV
- n=504
- n=33
- p<0.001

**E** OS-Mayo cohort

- NGS detected
  - No MYC abnormality
  - MYC SV
- n=92
- n=40
- p=0.990

**F** PFS-Mayo cohort

- NGS detected
  - No MYC abnormality
  - MYC SV
- n=85
- n=33
- p=0.490

**G** OS-CoMMPass cohort

- NGS detected
  - No MYC abnormality
  - MYC SV
- n=225
- n=320
- p=0.415

**H** PFS-CoMMPass cohort

- NGS detected
  - No MYC abnormality
  - MYC SV
- n=318
- n=224
- p=0.755
Figure 4

A. OS-CoMMpass cohort

B. PFS-CoMMpass cohort

p=0.003

p<0.001
Clinical Cancer Research

The prognostic role of MYC structural variants identified by NGS and FISH in multiple myeloma

Neeraj Sharma, James B Smadbeck, Nadine Abdallah, et al.

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